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Original Article

Cloning and primary characterizations of rLcn9, a new member of epididymal lipocalins in rat

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Lipocalins are a structurally conserved and diversely functional family of proteins that are of potential importance in epididymis functions. The rat Lcn9 gene was cloned by *in silico* methods and genome walking based on homology to the rhesus monkey epididymal ESC513 and its polyclonal antisera were prepared. The rat Lcn9 gene is located on chromosome 3p13 spanning 7 exons, contains 2.3 kb and encodes 179 amino acids with a 17-amino acid signal peptide. Northern blot, western blot, and immunohistochemical staining analysis revealed that rat Lcn9 was a novel epididymis-specific gene, expressed selectively in the proximal caput region, influenced by luminal fluid testicular factors. Moreover, Lcn9 protein was modified by *N*-glycosylation and bound on the postacrosomal domain of caput sperm. In conclusion, the rat Lcn9 exhibited tissue-, region-, and temporal-specific expression patterns and its expression was regulated by luminal testicular factors. Its potential roles in sperm maturation are discussed.

Keywords epididymis; lipocalin; Lcn9 gene

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Introduction

The epididymis, once viewed as useless, is now understood to perform the vital functions of sperm maturation, transportation, protection, and storage [1]. A gradient of differentiation characterizes the epididymal tubule epithelium that varies from the tall columnar principal cells of the caput to the shorter corpus and the much shorter caudal epithelium. The accompanying regional differences in gene expression are keys to the postnatal development and mature function of the epididymis. The epididymal epithelium, by blocking exchange with the blood and by active secretion and absorption, establishes gradients in the tubule lumen of proteins and other solutes that are critical for sperm maturation and storage [2,3]. Gene expression in these highly differentiated cells is region specific so that different region serves different functions [4].

The importance of epididymal sperm maturation is clinically highlighted by the fact that a high percentage of male infertility in humans originates from the malfunction of the epididymis [5]. For this reason, the epididymis was proposed as an ideal target organ for the development of male contraceptives by means that would avoid damage to the germ line [6]. Accordingly, many epididymal secretory proteins have been identified and characterized [4]. However, only a few proteins have been identified with direct involvement in sperm maturation, including Bin1b in acquisition of sperm motility as well as host defense in reproductive tract [7,8], DEFB126 in capacitation [9–11], HE6 in reabsorption [12], and Crisp-1 in sperm-egg fusion and capacitation [13,14]. Notwithstanding these important contributions, our understanding of involvement of epididymal proteins in sperm maturation is far from complete.

Previously, we cloned 11 epididymal-specific new genes in *Macaca mulatta* [15]. Among them, two genes (ESC513 and ESC384) revealed homology to lipocalins, so they are named as lipocalin 9 (Lcn9) and 8 (Lcn8), respectively. Experimental studies in primates are largely precluded for obvious ethical and practical reasons [16]. Thus, we searched for their orthologs in the rat.

The lipocalin family of ancient and structurally conserved hydrophobic ligand-binding proteins is a diverse

family present in all the major taxonomic groups from prokaryotes to plants, invertebrates, and vertebrates [17]. Lipocalins, which have been classified as transport proteins in the past, exhibit great functional diversity, with roles in taste and odor chemoreception, coloration, immune modulation, prostaglandin D synthesis, and several aspects of cell regulation including receptor-mediated induction of apoptosis [18]. Hitherto, it has been demonstrated that most lipocalin genes showed ubiquitous distribution in a wide range of tissues. However, it has been reported so far that only seven lipocalins were expressed specifically in the epididymis [19–21]. These epididymal lipocalins also showed region-specific expression [22], suggestive of region-specific function and potential involvement in sperm maturation [23].

Only two of the rat orthologs of these epididymis-specific lipocalins, E-RABP (Lcn5) and EP17 (Lcn8), have been investigated [21,24,25]. The mouse orthologue of Lcn9 was described at the genomic and mRNA levels [19]. Herein we report the cloning of the cDNA for the rat lipocalin gene Lcn9 (GenBank accession No. DQ537496), and evaluate its expression at both mRNA and protein levels as a first step toward a more complete understanding of its biological importance in epididymis.

Materials and Methods

Animals

Healthy adult male SD (Sprague-Dawley) rats (about 300–400 g) and male New Zealand white rabbits (about 2.5 kg), which were both purchased from the Animal Center of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), were raised in the animal house of the institute for an additional 7–10 days before manipulation. They were given food and water regularly throughout the experiments. All experiments were conducted in accordance with the Institute Animal Care Committee of the Shanghai Institutes for Biological Sciences, with internationally accepted guidelines for the humane care and use of laboratory animals.

cDNA and protein sequence analysis

The rat Lcn9 orthologue of monkey ESC513 was obtained using the monkey protein sequence as a query in a BLASTN search against rat genomic sequence. Based on this orthologous rat sequence, two primers were designed (forward primer, 5′-ATGGCCTCAGACAACATG-3′, and the reverse primer, 5′-TGGAAGATGGCATACATT-3′) and used to amplify a 245-bp cDNA fragment by reverse transcription-polymerase chain reaction (RT-PCR) according to the manufacturer's recommendations (TaKaRa, Dalian, China). The cDNA fragment was verified by automated sequencing, and then used as a probe to screen the rat epididymal cDNA library to obtain the 824 bp full-length cDNA.

The percent identities of amino acid sequence analysis were determined using the Clustal W Method of DNAStar software. The signal peptide cleavage sites, *N*-glycosylation sites, phosphorylation sites, and ubiquitination sites were, respectively, predicted at the website http://www.cbs.dtu.dk/services/SignalP by SiganlP.3.0 Server, http://www.cbs.dtu.dk/services/NetNGlyc/ by NetNGlyc 1.0 Server, http://www.cbs.dtu.dk/services/NetPhos/ by NetPhos 2.0 server, and http://www.abgent.com/doc/sumoplot by SUMOplot™.

RNA isolation and northern blot analysis

Tissue samples excised from male rats were frozen immediately in liquid nitrogen. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendations [26]. The total RNA of each sample was separated by 1.5% agarose gel and blotted to Hybond N^+ membrane (Amersham Biosciences, Piscataway, USA). A ^{32}P-labeled 245 bp cDNA fragment of rLcn9 was used as a probe, and an 18S rRNA hybridization signal was served as a control. Autoradiographs showing pronounced differences in expression were analyzed by densitometry. The relative intensity of hybridization was investigated using the software Gelwork3.01.

Castration and androgen replacement

As described previously [27], 120-day-old normal male SD rats were castrated bilaterally by sodium pentobarbital anesthesia. Animals were divided into nine groups (4–7 rats per group) and killed on Days 0, 1, 3, 5, and 7 after castration as well as 1, 3, 5, and 7 days after the initial testosterone propionate injection. The epididymides were excised and used for RNA extraction. Androgen supplementation began on the seventh day after castration, and rats were injected with testosterone propionate (3 mg/kg body weight) every 2 days. Pooled serum samples for each group were sent to the Shanghai Zhongshan Hospital (Shanghai, China) for the measurement of testosterone concentration by radioimmunoassay (RIA).

Preparation of polyclonal antibodies

The rat Lcn9 cDNA fragment encoding mature peptide of 162 amino acids excluding the signal peptide was amplified by PCR with the forward primer, GCTAGCCAGT TCAACTTGCACATTG (*Nhe*I underlined), and the reverse primer, AAGCTTCTGGCTCCTCTTAACGTCTA (*Hin*dIII underlined), at denaturing, annealing, and extension temperatures of 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s, respectively, using Ex-Taq (TaKaRa). This fragment was inserted into a T-easy vector (Promega, Madison, USA). After digested by *Nhe*I and *Hin*dIII, it was inserted into a pET 28a (+) vector (Novagen, Gibbstown, USA).

The expression was performed following the standard recommendations in the pET expression manual (Novagen). The recombinant protein was induced by isopropylthiogalactoside in the strain *Escherichia coli* DE3. The purification of the recombinant protein from inclusion bodies was executed as described previously [27]. The polyclonal antibodies against rat Lcn9 mature protein were produced according to the one-month-procedure developed previously by our lab [28]. The specificity of prepared antisera was confirmed by western blot analysis of the recombinant protein and protein extract was isolated from different tissues.

Protein extracts and western blot analysis

Protein extraction and western blot analysis were performed as described earlier [27] with a minor modification. Total protein extracts for each sample (20 μg) were separated on 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and semidry blotted to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, San Francisco, USA). The polyclonal antisera against rat Lcn9 recombinant protein were used as the primary antibody (dilution 1 : 10,000). The second antibody was a goat horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) (dilution 1 : 10,000; CalBiochem, San Diego, USA).

N-glycanase digestion

The peptide *N*-glycanase F (New England Biolabs, Ipswich, USA) treatment was performed to remove the carbohydrate from the native protein according to the manufacturer's instructions. The total epididymal caput tissue proteins (40 μg) from a sexually mature male rat were digested at 37°C for 42 h. The control sample of 18 μg caput protein was incubated without enzyme. The *N*-glycosylation modification of rat Lcn9 was then evaluated by western blot analysis with the primary Lcn9 and secondary HRP-conjugated anti-rabbit IgG antibodies diluted 1 : 5000, respectively.

Immunohistochemical staining analysis

Immunohistochemical staining (IHC) of antibody against rat Lcn9 was done with 120 days rat epididymis tissue using ABC kit (Sino-American Biotechnology Company, Luoyang, China). The procedure of investigation was followed according to the manufacturer's directions. The anti-rat LCN9 antibody (1 : 600) was incubated overnight at 4°C, and the HRP-conjugated goat anti-rabbit IgG (1 : 200) was used for 1 h at room temperature. As a negative control, serial sections were subjected to the same manipulation, with pre-immune serum replacing the primary antibody. The sections were observed by an optical Olympus microscope (Tokyo, Japan).

Immunofluorescence staining analysis

Immunofluorescence detection of proteins associated with spermatozoa was described previously [27]. The epididymis was excised into three segments of the caput, corpus, and cauda. Spermatozoa from these segments were collected and rinsed with phosphate-buffered saline (PBS), and then placed on polylysine-coated slides. The slides were air dried and stored. The staining procedure was performed using rabbit anti-rat Lcn9 antisera as primary antibody (dilution 1 : 200) and fluorescein isothiocyanate-conjugated anti-rabbit IgG as a secondary antibody (dilution 1 : 200). Immunofluorescent staining was observed and photographed using an Olympus microscope.

Results

Cloning of rat rLcn9 cDNA

The Lcn9 gene is located in the lipocalin gene-rich region of rat chromosome 3p13 and near the epididymal lipocalin genes Lcn5, Lcn8, Lcn10, Lcn12, and Lcn13, which orthologs in mouse have been shown to be expressed only in the epididymis [19]. Lcn9 spans 2347 bp with seven exons and six introns, a conserved lipocalin gene structure. The entire 540-bp open reading frame (ORF) encoding a protein of 179 amino acids with a predicted size of 20.56 kDa is located in the second exon through the sixth exon [**Fig. 1(B)**]. The N-terminal 17 amino acids form a predicted signal peptide, and the cleavage of this peptide would produce a mature protein of 162 amino acids with a calculated molecular mass of 18.85 kDa [**Fig. 1(A)**]. Nine potential sites of post-translational modification are predicted in this sequence: three *N*-glycosylation sites at N47, N68, N130; four phosphorylation sites at Ser35, Ser97, Thr172, and Tyr117; and two ubiquitination sites at K129 and K54 [**Fig. 1(A)**].

The rat Lcn9 protein is 74.2% identical to mouse Lcn9, but it has extremely low identity (4.6%–28.7%) to other rat lipocalins. However, all rat lipocalins encode proteins containing the conserved lipocalin motifs (G-X-W, T-D-Y, and K/R) as well as a putative signal peptide and the cysteine residues that are involved in the formation of a disulfide bond (**Fig. 2**). It is typical in the lipocalin family that overall sequence similarity among various members is low but they shared the conserved sequence motifs and high similarities of three-dimensional structure, so that they can perform similar function of transporting hydrophobic small molecules.

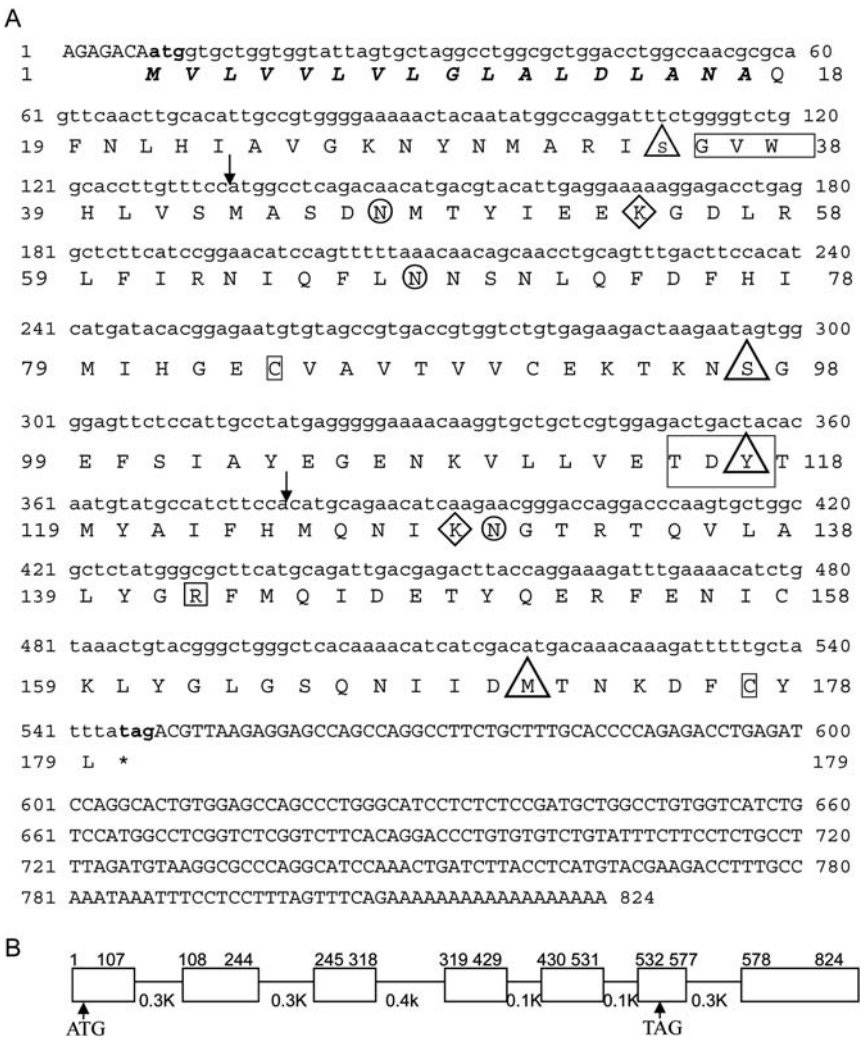

Figure 1 Rat Lcn9 gene structure and cDNA and protein sequence (A) The rLcn9 open reading frame (ORF) contains 540 bp coding for a 179-amino-acid protein, and the initial and terminal codons are indicated in bold. The protein contains conserved motifs (GVW, TDY, R, and C) indicated by '□'; a putative signal peptide (italics and bold) with a cleavage site between amino acids 17 and 18; three *N*-glycosylation sites (N47, N68, N130) indicated by '○'; three serine, threonine, and tyrosine phosphorylation sites (S35, S97, T172, Y117) indicated by '△'. Two ubiquitination sites (K129 and K54) indicated by '◇'. Sequences between two arrows (134−378 bp) were used as the probe for the northern blot analysis. (B) The rat LCN9 gene contains 7 exons represented by open boxes. The nucleotides in the cDNA are indicated by the numbers above the boxes. The numbers between the boxes indicate the sizes of the introns.

Epididymis-specific expression of rat Lcn9 mRNA

Total RNAs from three epididymal regions (caput, corpus, and cauda) and other tissues of adult male rats were analyzed by northern blot analysis (**Fig. 3**). A strong hybridizing signal (about 1.1 kb) was seen exclusively in the caput region of rat epididymis. No mRNA was detected in the other regions of the epididymis or in other tissues examined. This result suggested a potential role for Lcn9 protein related to sperm maturation.

Regulation of rat rLcn9 mRNA by non-androgen testicular factors

Epididymal gene expression can be modulated by testis via circulating testis androgens and/or luminal testicular factors. Testicular regulation of the Lcn9 gene was analyzed in the bilateral orchiectomized rat with and without testosterone supplementation (**Fig. 4**). As expected, the serum testosterone level diminished rapidly and reached the limits of detection from the first day after gonadectomy. In parallel, Lcn9 expression sharply declined on the first day, with no signal detected from the third to seventh day after surgery. Testosterone replacement for the animals 7 days after castration caused a steep increase in the serum testosterone concentration, but the Lcn9 mRNA in the epididymis did not follow the same trend as the serum testosterone level. Instead, Lcn9 remained undetected, suggesting the involvement of testicular fluid factors.

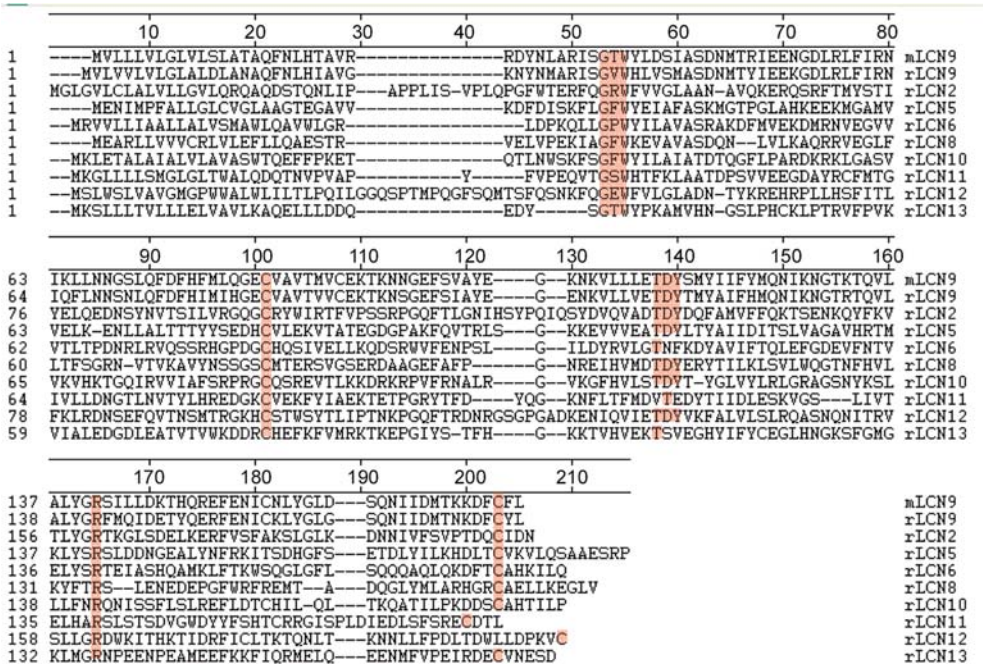

Figure 2 Amino acid sequences alignment of rat lipocalin genes (Lcn2, Lcn5, Lcn6, and Lcn8–Lcn13) with mouse Lcn9 Dashes show alignment gaps. The conserved motifs (G-X-W, T-D-Y, R, and C) of the lipocalin family are indicated by red color. The putative signal peptides are indicated by blue color. Amino acid residue numbers are shown above and on the left.

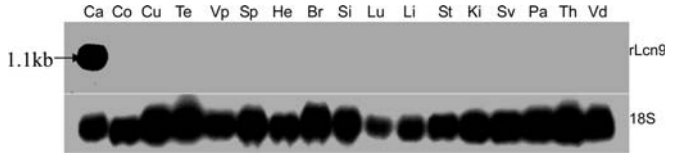

Figure 3 Selective expression of Lcn9 mRNA in the rat epididymis Northern blot analysis shows the cn9 mRNA is highly expressed in the caput region, but not in the other regions or tissues. Ca, caput; Co, corpus; Cu, cauda; Te, testis; Vp, ventral prostate; Sp, spleen; He, heart; Br, brain; Si, small intestine; Lu, lung; Li, liver; St, stomach; Ki, kidney; Sv, seminal vesicle; Pa, pancreas; Th, thyroid; Vd, vas deference.

Preparation of polyclonal antisera against Lcn9 and investigation on *N*-glycosylation of Lcn9 endogenous protein

The sensitivity of the prepared rabbit polyclonal antisera raised against the purified recombinant Lcn9 protein is quite high allowing the detection of 0.5 ng antigen [**Fig. 5(A)**]. For validating the specificity of these antibodies, we investigated various adult male rat tissues and different regions in the epididymis by western blot analysis [**Fig. 5(B)**]. A single band of 27-kDa protein was observed exclusively in extracts from the caput region of epididymis and not in other regions or tissues examined [**Fig. 5(B)**]. No signal was obtained with the pre-immune rabbit serum (data not shown). These results were agreed with those of mRNA analysis [**Figs. 3** and **5(B)**].

It was not surprising that the apparent molecular mass of the 27-kDa target protein detected by western blot was higher than predicted one (20.56 kDa for the full-length protein and 18.85 kDa for the mature peptide). Since there are three putative *N*-glycosylation sites in this protein, an *N*-deglycosylation test was carried out using *N*-glycanase. As a result, a decreased molecular mass similar to the theoretical size of the mature form of rat rLcn9 was detected [**Fig. 5(C)**], suggesting that a post-translational carbohydrate addition accounts for these differences.

Regional, temporal, and cell-specific expressions of Lcn9 protein in the epididymis

For gaining further insight into Lcn9 protein potential function in male reproduction, immunohistochemical analysis was performed in rat epididymis (**Fig. 6**). Strong positive signals were observed in the proximal caput (also called initial segment) but not in corpus and cauda regions [**Fig. 6(A)**]. However, the signal in each sub-region of the caput was not uniform. Instead, some sub-regions strongly expressed Lcn9 and some weakly expressed it [**Fig. 6(B)**]. Among epithelia cells in the same region, not all the cells demonstrated the intense immunostaining. Only certain cells showed significant immunoreactivity in several ducts [**Fig. 6(C3)**] with a banded expression pattern. Compared with the epithelial cells showing faint expression, the lumen in some ducts showed strong signals. Luminal spermatozoa were all strongly positive for Lcn9.

To determine whether Lcn9 could be involved in developmental as well as mature functions, localization of Lcn9 was analyzed in the tissues from animals of different

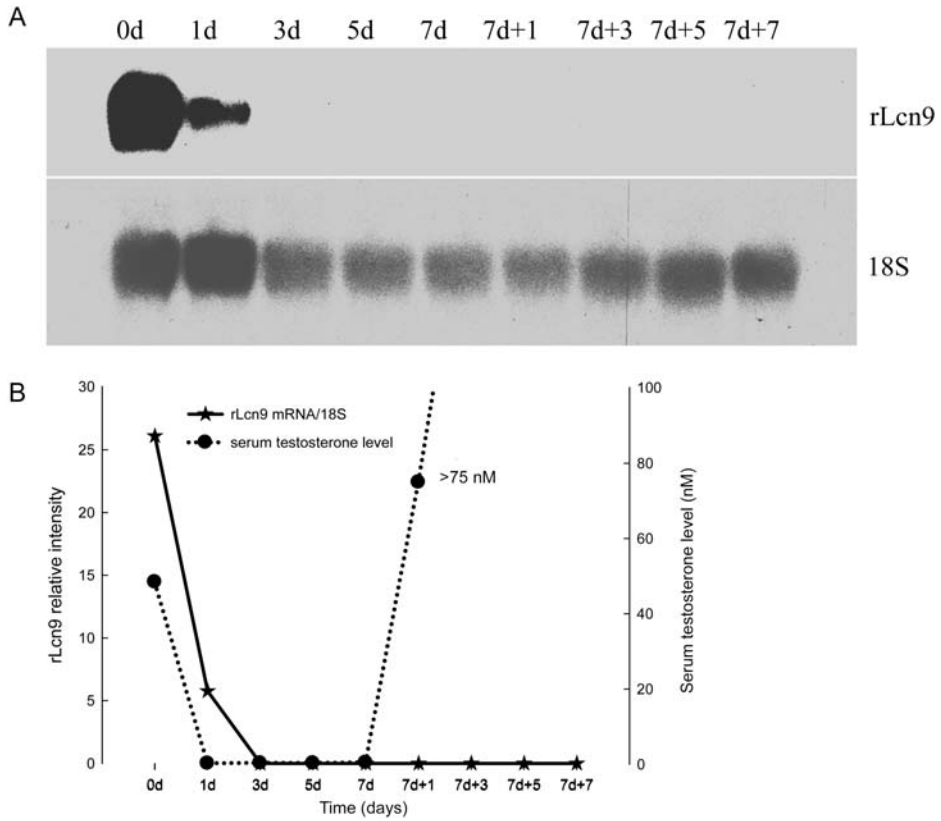

Figure 4 Lcn9 expression after androgen ablation (A) Northern blot analysis. 0d was prior to castration; 1d, 3d, 5d, and 7d indicated days after bilateral castration; and 7d+1, 7d+3, 7d+5, and 7d+7 indicated days after the initial injection of testosterone propionate applied to the 7d-castrated rats. Injections were continued every 2 days. (B) Comparison of Lcn9 mRNA and the serum testosterone levels. Left *Y* axis is hybridization density of Lcn9 mRNA/18S ribosomal RNA in the rat epididymis, while right *Y* axis is the serum testosterone level (expressed in nM). The RNAs were pooled from four to seven animals per group.

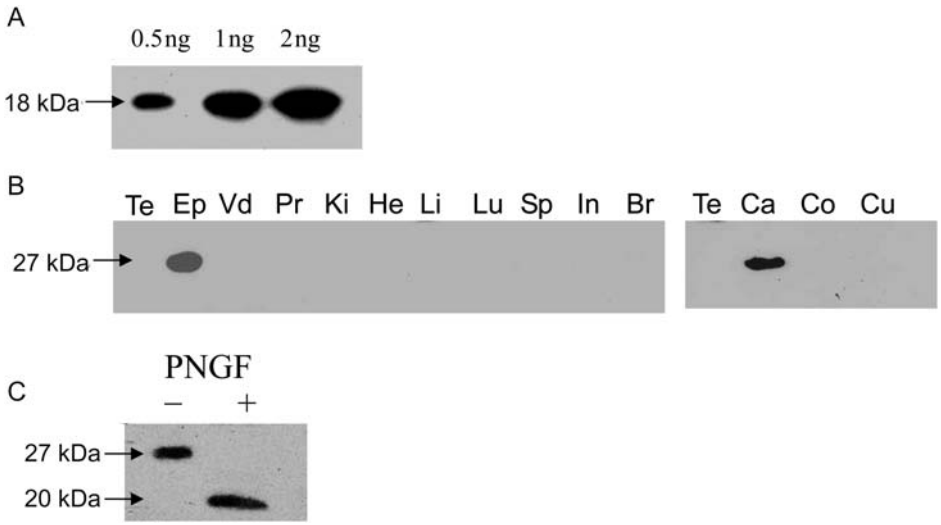

Figure 5 Western blot analysis of rat rLcn9 recombinant protein and native protein (A) The sensitivity analysis of rLcn9 antibody with 0.5, 1, and 2 ng of the antigen peptide. (B) The specificity analysis of rLcn9 antibody with rat native protein. Ca, caput epididymidis; Co, corpus epididymidis; Cu, cauda epididymidis; Te, testis; Ep, epididymis; Vd, vas deferens; Pr, prostate; Ki, kidney; He, heart; Li, liver; Lu, lung; Sp, spleen; In, intestine; Br, brain. (C) The change of molecular masses of rat rLcn9 before and after deglycosylation by PNGase-F.

ages using immunohistochemistry (**Fig. 7**). The Lcn9 protein began to be detected at the age of 45 days; after that, the expression increased gradually and remained at a high level with strong immunoreactivity restricted in the epithelial cells of the proximal caput/initial segment region (**Fig. 7**).

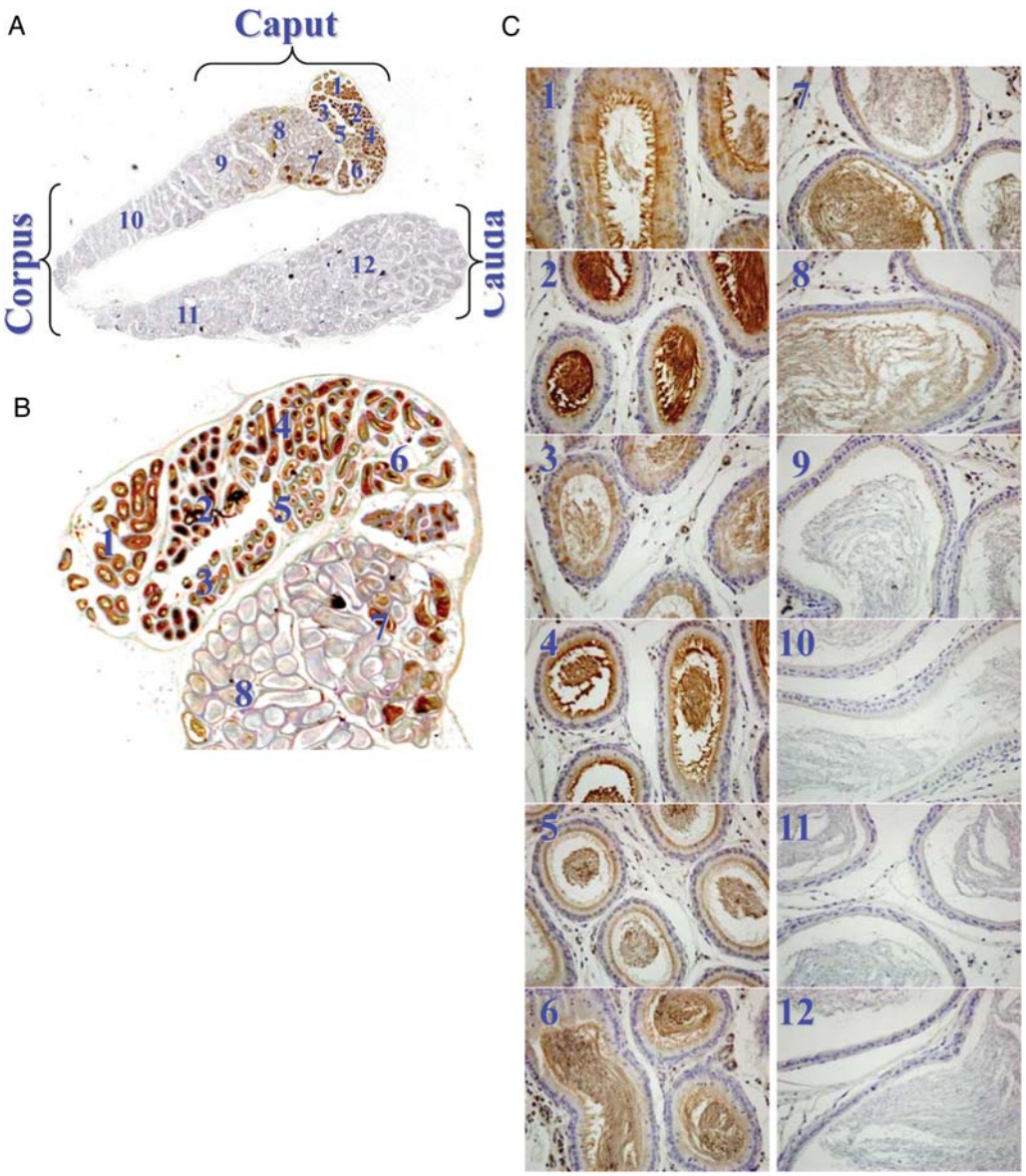

Figure 6 The localization of rLcn9 in the 120-day-old rat epididymis by immunohistochemistry (A) The rat rLcn9 localization in different regions of the epididymis. The photograph is from the scanning of the section by Magicscan V4.6. (B) The expression pattern of rLcn9 in the whole rat epididymis. The magnified photograph of the caput region in (A). (C) The magnified photographs of some parts of (A): 1–9, caput; 10, proximal corpus; 11, distal corpus; 12, cauda. Magnification, ×40. (Note: parts here are corresponding to segments reported before [38]: part 1 to segment 1, parts 2 and 3 to segment 2, parts 4 and 5 to segment 3, part 6 to segment 4.)

Immunohistochemistry suggested that Lcn9 was associated with spermatozoa in the lumen. To make further confirmation spermatozoa were respectively isolated from the caput, corpus, and cauda epididymis and stained using immunofluorescence. No immunofluorescence was visualized on formaldehyde-fixed or unfixed spermatozoa from either the corpus or the cauda epididymis (data not shown). Immunoreactive Lcn9 was concentrated over the post-acrosomal region of unfixed spermatozoa from caput epididymidis [**Fig. 8(B,C)**]. The negative control (pre-immune rabbit serum) did not show any Lcn9 immunoreactivity on spermatozoa from the three regions of epididymis [**Fig. 8(A)**].

Discussion

In the present study, we identified and characterized the rat Lcn9 gene in the epididymis at both mRNA and protein levels. We found that rat Lcn9 was exclusively expressed in the caput region, and regulated by luminal testicular factors. These results were in agreement with those of mLcn9 in mouse at the mRNA level [19]. It remains to be further investigated whether other results of rat Lcn9 is consistent with those of mouse Lcn9. Intriguingly, the results of rLcn9 expression are basically similar to another lipocalin mLcn8 in mouse [19,21,29]. However, there are several

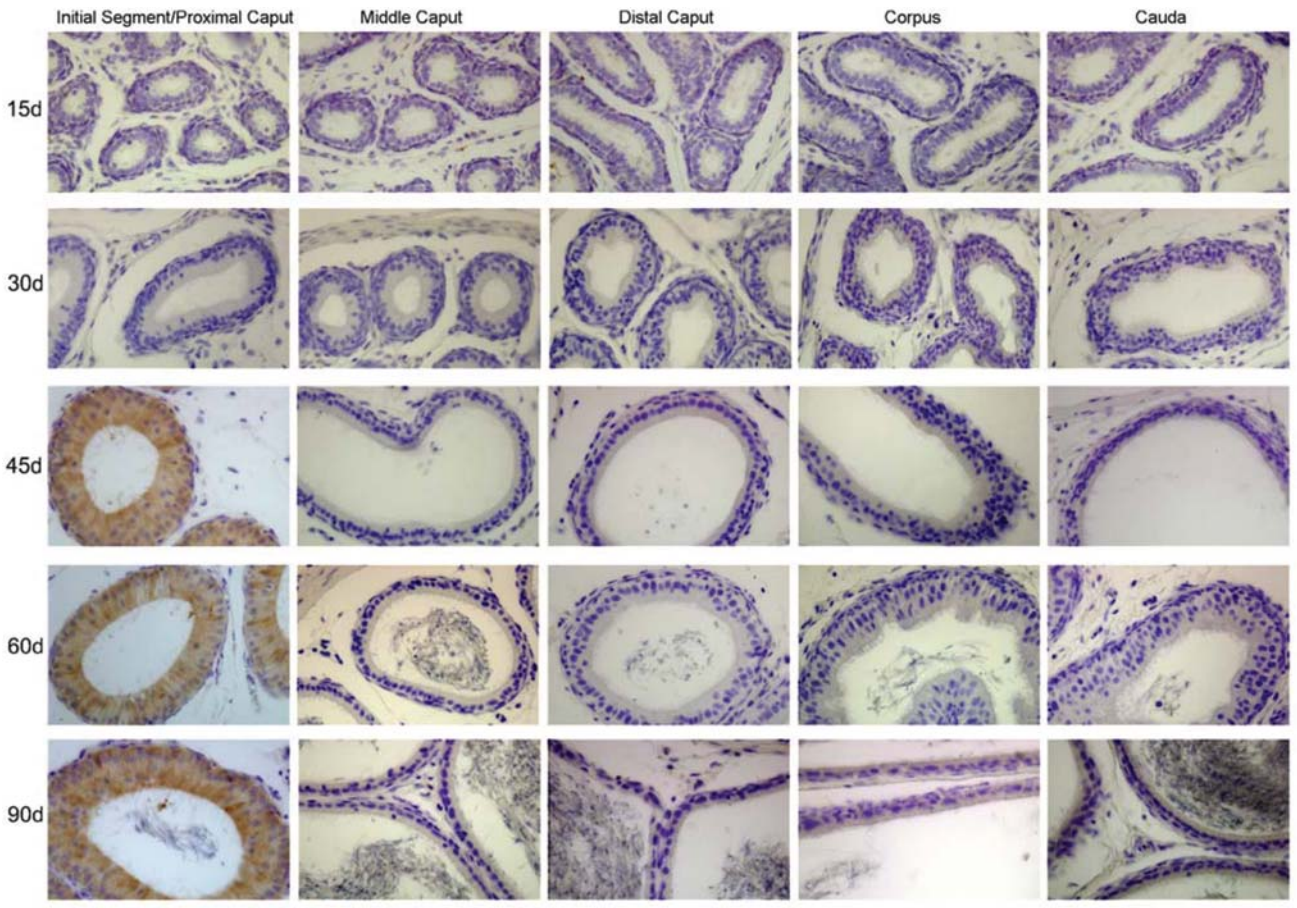

Figure 7 The rat LCN9 localization in different regions of the rat epididymis sections from different developmental stages by immunohistochemistry The ages (expressed in days) are shown on the left, and the regions of the epididymis are shown at the top.

differences: one is that mLcn8 localized at the supranuclear region of the epithelial cells, while rLcn9 at the cytoplasm; the other is that mLcn8 protein showed no sperm binding, whereas rLcn9 protein showed strong luminal signals and significant sperm association. Compared with rLcn9, the other epididymis-specific lipocalin genes (besides mLcn8 and 9) investigated so far showed some different or similar features. For expressional regions, mLcn10 as well as mLcn8 and 9 [19] was expressed in proximal caput/initial segment; mLcn5 [30,31] in mid and distal caput; rat ESP I (the ortholog gene of mLcn5) [32], and the human Lcn6 [20] in the caput region; the mouse 24p3 (mLcn2) in the caput and corpus [33]; the mouse prostaglandin D_2 synthase (PGDS) in the distal caput, corpus, and cauda [34]. For postnatal development, mLcn10 and mLcn13 as well as mLcn8 and mLcn9 were not detectable before 3 weeks, and increased from 3 to 4 weeks of age [19,21]; mLcn2, mLcn5, mLcn12, and mPGDS persisted before 3 weeks and increased thereafter [19]. For expression regulation, mLcn13 besides mEP17and mLcn9 in mouse were under the control of luminal testicular factors [19,21]; PGDS [35], mLcn2, mLcn5 and mLcn12 were regulated by circulating androgens; whereas mLcn10 gene was modulated by this two kinds of testicular factors [19]. For glycosylation modification, the mouse PGDS as well as mLcn5 are *N*-glycosylated [21,34]. For cell localization, E-RABP and L-PGDS were localized to principal cells in epididymis [36]; mEP17 in principal cells and clear cells. For sperm binding, L-PGDS [37], 24p3 [33], and Lcn6 [20], were associated with spermatozoa in epididymis. According to all the above-mentioned investigations, the expressional features of rLcn9 were not completely identical to those of the other lipocalin genes, which suggested that rLcn9 has an important but non-redundant function in male fertility, although functions of all the other lipocalins in epididymis are still not known.

Based on the histological appearance and anatomical structure, we divided rat epididymis into the 19 connective tissue-bordered segments (**Fig. 7**) as previously described [38]. Here, we found that the Lcn9 signals present in segments 2 and 3 were not uniform. Rather, segments 2 and 3 contained connective tissue septa that further subdivide them into two subsegments, one with a strong signal and the other a weak one. Previous studies have shown that these connective tissue septa restrict movement of paracrine

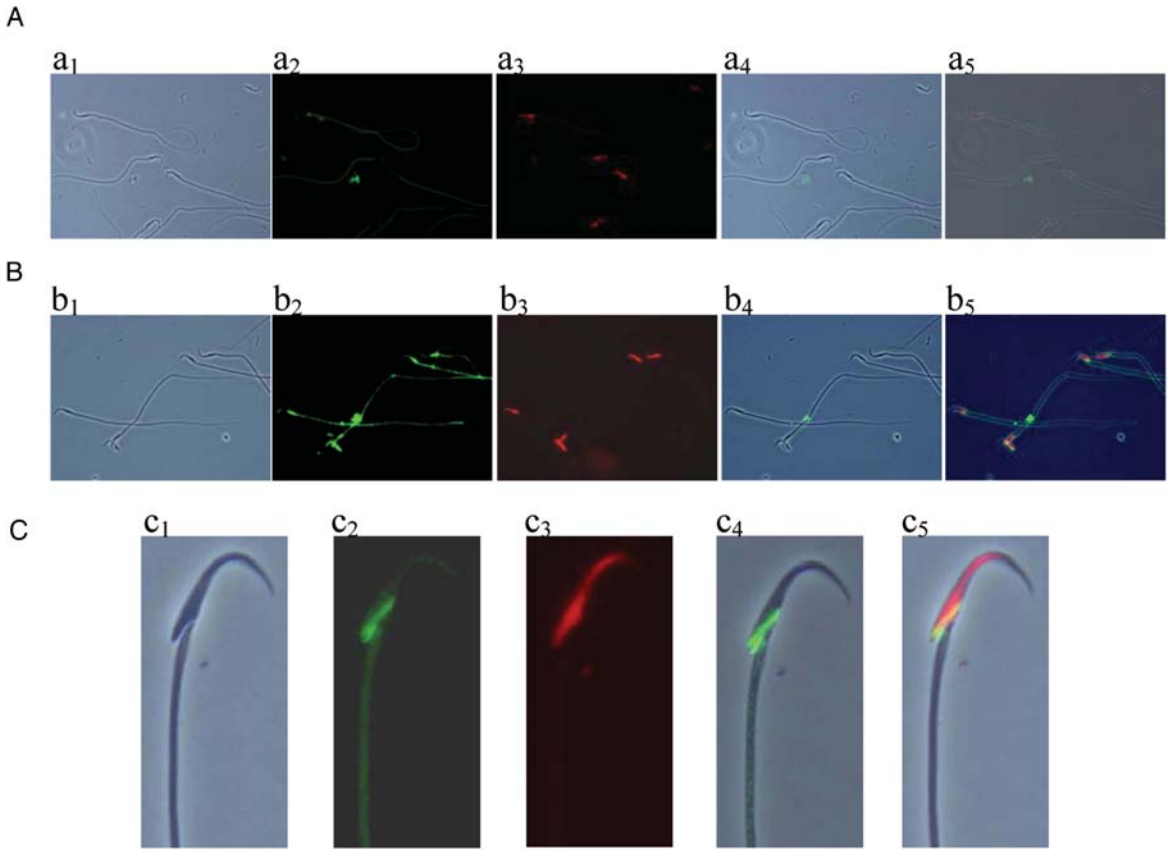

Figure 8 Lcn9 localization on the caput spermatozoa by immunofluorescence (A) Immunolocalization of rat Lcn9 on spermatozoa isolated from the caput epididymal region by pre-immune sera. (B) Immunolocalization of rat Lcn9 on spermatozoa isolated from the caput epididymal region by rLcn9 antibodies. (C) Magnified heads of spermatozoa in (B). Magnification, ×100. (a1, b1, c1) Phase contrast view of sperm in a2, b2, c2; (a2, b2, c2) The immunofluorescence of rat rLcn9 (FITC labeled); (a3, b3, c3) The nuclear (PI); (a4, b4, c4) The merged photograph of a1 and a2, b1 and b2, and c1 and c2, respectively; (a5, b5, c5) The merged photograph of a3 and a4, b3 and b4, and c3 and c4, respectively.

factors allowing differential control of gene expression in adjacent segments and fine functional specialization [39]. Our results suggest that Lcn9 expression is also regulated by paracrine factors.

In addition, to determine the function of rLcn9, other approaches are under way, including investigations to achieve the active recombinant rat rLCN9 protein and to analyze the reproductive phenotype of the Lcn9 knockdown rat and knockout mouse.

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References

1 Banks S, King SA, Irvine DS and Saunders PT. Saunders. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. Reproduction 2005, 129: 505–514.
2 Dacheux JL. Protein secretion in the epididymis. In: Robaire B and Hinton BT, eds. The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers, 2002, 151–168.
3 Jones R. Plasma membrane composition and organisation during maturation of spermatozoa in the epididymis. In: Robaire B and Hinton BT, eds. The Epididymis: from Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers, 2002, 405–416.
4 Dacheux JL, Gatti JL and Dacheux F. Contribution of epididymal secretory proteins for spermatozoa maturation. Microsc Res Tech 2003, 61: 7–17.
5 Khole V. Epididymis as a target for contraception. Indian J Exp Biol 2003, 41: 764–772.
6 Reyes A and Chavarria ME. Interference with epididymal physiology as possible site of male contraception. Arch Androl 1981, 7: 159–168.
7 Zhou CX, Zhang YL, Xiao L, Zheng M, Leung KM, Chan MY and Lo PS, *et al*. An epididymis-specific beta-defensin is important for the initiation of sperm maturation. Nat Cell Biol 2004, 6: 458–464.

8 Li P, Chan HC, He B, So SC, Chung YW, Shang Q and Zhang YD, *et al*. An antimicrobial peptide gene found in the male reproductive system of rats. Science 2001, 291: 1783–1785.

9 Yudin AI, Tollner TL, Li MW, Treece CA, Overstreet JW and Cherr GN. ESP13.2, a member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. Biol Reprod 2003, 69: 1118–1128.

10 Tollner TL, Yudin AI, Treece CA, Overstreet JW and Cherr GN. Macaque sperm release ESP13.2 and PSP94 during capacitation: the absence of ESP13.2 is linked to sperm-zona recognition and binding. Mol Reprod Dev 2004, 69: 325–337.

11 Yudin AI, Generao SE, Tollner TL, Treece CA, Overstreet JW and Cherr GN. Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. Biol Reprod 2005, 73: 1243–1252.

12 Davies B, Baumann C, Kirchhoff C, Ivell R, Nubbemeyer R, Habenicht UF and Theuring F, *et al*. Targeted deletion of the epididymal receptor HE6 results in fluid dysregulation and male infertility. Mol Cell Biol 2004, 24: 8642–8648.

13 Roberts KP, Johnston DS, Nolan MA, Wooters JL, Waxmonsky NC, Piehl LB and Ensrud-Bowlin KM, *et al*. Structure and function of epididymal protein cysteine-rich secretory protein-1. Asian J Androl 2007, 9: 508–514.

14 Cohen DJ, Da Ros VG, Busso D, Ellerman DA, Maldera JA, Goldweic N and Cuasnicu PS. Participation of epididymal cysteine-rich secretory proteins in sperm-egg fusion and their potential use for male fertility regulation. Asian J Androl 2007, 9: 528–532.

15 Liu Q, Hamil KG, Sivashanmugam P, Grossman G, Soundararajan R, Rao AJ and Richardson RT, *et al*. Primate epididymis-specific proteins: characterization of ESC42, a novel protein containing a trefoil-like motif in monkey and human. Endocrinology 2001, 142: 4529–4539.

16 Kirchhoff C. Specific gene expression in the human and non-human primate epididymism. In: Robaire B and Hinton BT, eds. The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers, 2002, 201–218.

17 Grzyb J, Latowski D and Strzalka K. Lipocalins—a family portrait. J Plant Physiol 2006, 163: 895–915.

18 Flower DR. The lipocalin protein family: structure and function. Biochem J 1996, 318(Part 1): 1–14.

19 Suzuki K, Lareyre JJ, Sanchez D, Gutierrez G, Araki Y, Matusik RJ and Orgebin-Crist MC. Molecular evolution of epididymal lipocalin genes localized on mouse chromosome 2. Gene 2004, 339: 49–59.

20 Hamil KG, Liu Q, Sivashanmugam P, Anbalagan M, Yenugu S, Soundararajan R and Grossman G, *et al*. LCN6, a novel human epididymal lipocalin. Reprod Biol Endocrinol 2003, 1: 112.

21 Fouchecourt S, Lareyre JJ, Chaurand P, DaGue BB, Suzuki K, Ong DE and Olson GE, *et al*. Orgebin-Crist, identification, immunolocalization, regulation, and postnatal development of the lipocalin EP17 (epididymal protein of 17 kilodaltons) in the mouse and rat epididymis. Endocrinology 2003, 144: 887–900.

22 Suzuki K, Yu X, Chaurand P, Araki Y, Lareyre JJ, Caprioli RM and Matusik RJ, *et al*. Epididymis-specific promoter-driven gene targeting: a transcription factor which regulates epididymis-specific gene expression. Mol Cell Endocrinol 2006, 250: 184–189.

23 Axner E. Sperm maturation in the domestic cat. Theriogenology 2006, 66: 14–24.

24 Newcomer ME. Structure of the epididymal retinoic acid binding protein at 2.1 A resolution. Structure 1993, 1: 7–18.

25 Zwain IH, Grima J and Cheng CY. Rat epididymal retinoic acid-binding protein: development of a radioimmunoassay, its tissue distribution, and its changes in selected androgen-dependent organs after orchiectomy. Endocrinology 1992, 131: 1511–1526.

26 Zhang J, Liu Q, Zhang W, Li J, Li Z, Tang Z and Li Y, *et al*. Comparative profiling of genes and miRNAs expressed in the newborn, young adult, and aged human epididymides. Acta Biochim Biophys Sin 2010, 42: 145–153.

27 Zhu CF, Liu Q, Zhang L, Yuan HX, Zhen W, Zhang JS and Chen ZJ, *et al*. RNase9, an androgen-dependent member of the RNase A family, is specifically expressed in the rat epididymis. Biol Reprod 2007, 76: 63–73.

28 Hu YX, Guo JY, Shen L, Chen Y, Zhang ZC and Zhang YL. Get effective polyclonal antisera in one month. Cell Res 2002, 12: 157–160.

29 Lareyre JJ, Winfrey VP, Kasper S, Ong DE, Matusik RJ, Olson GE and Orgebin-Crist MC. Gene duplication gives rise to a new 17-kilodalton lipocalin that shows epididymal region-specific expression and testicular factor(s) regulation. Endocrinology 2001, 142: 1296–1308.

30 Orgebin-Crist MC, Lareyre JJ, Suzuki K, Araki Y, Fouchécourt S, Matusik RJ and Ong DE. Retinoids and epididymal function. In: Robaire B and Hinton BT, eds. The Epididymis: From Molecules to Clinical Practice. New York: Kluwer Academic/Plenum Publishers, 2002, 339–352.

31 Lareyre JJ, Mattei MG, Kasper S, Ong DE, Matusik RJ and Orgebin-Crist MC. Genomic organization and chromosomal localization of the murine epididymal retinoic acid-binding protein (mE-RABP) gene. Mol Reprod Dev 1998, 50: 387–395.

32 Lareyre JJ, Zheng WL, Zhao GQ, Kasper S, Newcomer ME, Matusik RJ and Ong DE, *et al*. Molecular cloning and hormonal regulation of a murine epididymal retinoic acid-binding protein messenger ribonucleic acid. Endocrinology 1998, 139: 2971–2981.

33 Chu ST, Lee YC, Nein KM and Chen YH. Expression, immunolocalization and sperm-association of a protein derived from 24p3 gene in mouse epididymis. Mol Reprod Dev 2000, 57: 26–36.

34 Fouchecourt S, Chaurand P, DaGue BB, Lareyre JJ, Matusik RJ, Caprioli RM and Orgebin-Crist MC. Epididymal lipocalin-type prostaglandin D2 synthase: identification using mass spectrometry, messenger RNA localization, and immunodetection in mouse, rat, hamster, and monkey. Biol Reprod 2002, 66: 524–533.

35 Zhu H, Ma H, Ni H, Ma XH, Mills N and Yang ZM. Expression and regulation of lipocalin-type prostaglandin d synthase in rat testis and epididymis. Biol Reprod 2004, 70: 1088–1095.

36 Gerena RL, Eguchi N, Urade Y and Killian GJ. Stage and region-specific localization of lipocalin-type prostaglandin D synthase in the adult murine testis and epididymis. J Androl 2000, 21: 848–854.

37 Gerena RL, Irikura D, Eguchi N, Urade Y and Killian GJ. Immunocytochemical localization of lipocalin-type prostaglandin D synthase in the bull testis and epididymis and on ejaculated sperm. Biol Reprod 2000, 62: 547–556.

38 Jelinsky SA, Turner TT, Bang HJ, Finger JN, Solarz MK, Wilson E and Brown EL, *et al*. The rat epididymal transcriptome: comparison of segmental gene expression in the rat and mouse epididymides. Biol Reprod 2007, 76: 561–570.

39 Turner TT, Johnston DS, Jelinsky SA, Tomsig JL and Finger JN. Segment boundaries of the adult rat epididymis limit interstitial signaling by potential paracrine factors and segments lose differential gene expression after efferent duct ligation. Asian J Androl 2007, 9: 565–573.